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The influence of dicoumarol on the bioactivation of the carcinogen aristolochic acid I in rats

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Running Title: Dicoumarol and aristolochic acid *in vivo*

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Abstract

Aristolochic acid I (AAI) is the major toxic component of the plant extract aristolochic acid, which leads to the development of nephropathy and urothelial cancer in human. Individual susceptibility to AAI-induced disease might reflect variability in enzymes that metabolize AAI. *In vitro* NAD(P)H:quinone oxidoreductase (NQO1) is the most potent enzyme that activates AAI by catalyzing formation of AAI-DNA adducts which are found in kidneys of patients exposed to AAI. Inhibition of renal NQO1 activity by dicoumarol has been shown in mice. Here we studied the influence of dicoumarol on metabolic activation of AAI in Wistar rats *in vivo*. In contrast to previous *in vitro* findings dicoumarol did not inhibit AAI-DNA adduct formation in rats. Compared with rats treated with AAI alone, 11- and 5.4- fold higher AAI-DNA adduct levels were detected in liver and kidney, respectively, of rats pretreated with dicoumarol prior to exposure to AAI. Cytosols and microsomes isolated from liver and kidney of these rats were analyzed for activity and protein levels of enzymes known to be involved in AAI metabolism. The combination of dicoumarol with AAI induced NQO1 protein level and activity in both organs. This was paralleled by an increase in AAI-DNA adduct levels found in *ex vivo* incubations with cytosols from rats pretreated with dicoumarol compared to cytosols from untreated rats. Microsomal *ex vivo* incubations showed a lower AAI detoxication to its oxidative metabolite, 8-hydroxyaristolochic acid (AAIa), although cytochrome P450 (CYP) 1A was practically unchanged. Because of these unexpected results we examined CYP2C activity in microsomes and found that treatment of rats with dicoumarol alone and in combination with AAI inhibited CYP2C6/11 in liver. Therefore these results indicate that CYP2C enzymes might contribute to AAI detoxication.

Introduction

The herbal drug aristolochic acid (AA) derived from *Aristolochia* species is considered to be the cause of aristolochic acid nephropathy (AAN) (1-4), which is a rapidly progressive renal fibrosis, with a high risk for the patients of developing upper urothelial tract carcinoma and, subsequently, bladder urothelial carcinoma (4-5). Exposure to AA has also been linked to inhabitants of rural areas in the Balkans who develop nephropathy – Balkan endemic nephropathy (BEN) (4,7-9). Exposure of experimental animals to AA leads to characteristic AA-DNA adducts in renal tissue after reductive activation. The same DNA adducts, mainly 7-(deoxyadenosin-*N*⁶-yl)aristolactam I (dA-AAI) (Fig. 1), were detected in kidneys of AAN and BEN patients whereby their exposure to AA was identified (5,6,8,10-12). This deoxyadenosine adduct causes characteristic AT→TA transversions in critical genes of oncogenesis (e.g. *TP53* tumor suppressor gene) and such AT→TA mutations have indeed been found in urothelial tumors in AA-exposed humans (8,9,13-16), indicating a molecular mechanism associated with AA-induced carcinogenesis (7,17). AA has been classified as a Group I carcinogen by the International Agency for Research on Cancer.

AAI is activated by nitroreduction by both cytosolic and microsomal enzymes. Of the enzymes characterized so far, cytosolic NAD(P)H:quinone oxidoreductase (NQO1) was found to be the most active enzyme both in experimental animals and in human tissue (18-22) (Fig. 1). In microsomes from human liver, cytochrome P450 (CYP) 1A2 is the most active reductase followed by CYP1A1 and NADPH:CYP reductase (POR) (23-26). However, the two isoenzymes of the CYP1A family are mainly responsible for the oxidative demethylation of AAI to 8-hydroxyaristolochic acid I (aristolochic acid Ia, AAIA; Fig. 1) (24-27).

Previous studies with genetically engineered mice such as Hepatic P450 Reductase Null (HRN) mice which lack hepatic POR and therefore essentially all CYP-activity in hepatocytes, with *Cyp1a1*(-/-), *Cyp1a2*(-/-) and *Cyp1a1/1a2*(-/-) knockouts as well as with *CYP1A*-humanized mice demonstrated that the balance between reductive activation and

oxidative detoxication of AAI depends on the expression of both CYP1A1/1A2 and NQO1 (24-26,28). The knockout of enzymes which mainly detoxicate AAI was not the only reason for the observed higher DNA adduct levels, but a concomitant 3-fold increase in NQO1 protein levels in the livers of these mice was also important (26). Cytosolic fractions isolated from HRN mice activated AAI more efficiently to DNA adducts than hepatic cytosols of WT mice. These findings emphasized the importance of NQO1 not only for the activation of AAI *in vitro*, but also *in vivo*. AAI was also found to induce NQO1 protein levels and its enzyme activity in liver, kidney and lung of mice (26,28,29) and of Wistar rats treated with this compound (19). Again, higher NQO1 enzyme activity was associated with increased AAI-DNA adduct formation in *ex vivo* incubations of cytosols with AAI and DNA (26,28). Hence, AAI induces NQO1 and thereby its own metabolic activation leading to increased genotoxicity *in vivo*.

A role of NQO1 in renal AAI nitroreduction *in vivo* was proven in mice (male C57BL/6 mice) (22) in which the modulation of AAI metabolism by dicoumarol, an inhibitor of NQO1, was investigated. NQO1 activity was inhibited in dicoumarol-pretreated mice resulting in decreased levels of the reductive metabolite aristolactam I in kidney and increased amounts of AAI as well as AAIA in serum of AAI-exposed mice.

In the present study we investigated whether dicoumarol influences the genotoxicity of AAI in rats *in vivo*. In addition, the effect of this NQO1 inhibitor alone or in combination with AAI on enzymes metabolizing AAI (NQO1, CYP1A1/2) was investigated. DNA adduct formation by AAI was evaluated by the ³²P-postlabeling method *in vivo* and in *ex vivo* incubations using cytosols and microsomes isolated from kidneys and livers. Further, the AAI metabolite AAIA in these *ex vivo* incubations was measured by high pressure liquid chromatography (HPLC).

Materials and methods

Chemicals

NADPH, AAI (sodium salt), dicoumarol, Sudan I [1-(phenylazo)-2-hydroxynaphthalene], menadione (2-methyl-1,4-naphthoquinone) and calf thymus DNA were from Sigma Chemical Co (St Louis, MO, USA). 7-ethoxyresorufin and 7-methoxyresorufin were from Fluka Chemie AG (Buchs, Switzerland). Enzymes and chemicals for the ^{32}P -postlabeling assay were from sources described (23).

Animal experiments and sample preparation

The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which is in compliance with the Declaration of Helsinki. Animals were acclimatized for 5 days and maintained at 22°C with a 12 h light/dark period. Standardized diet and water were provided *ad libitum*. Groups of five weeks old male Wistar rats (~150 g, n = 3/group) were treated with dicoumarol, dissolved in sunflower oil. Dicoumarol was administered by gavage to rats twice at either 30 or 60 mg/kg body weight (bw), once at 3 p.m. and again the next day at 8 a.m. (total doses were 60 or 120 mg dicoumarol/kg bw). Another group of rats was injected *i.p.* with a single dose of AAI dissolved in 1% NaHCO_3 (20 mg/kg bw). In the study on the effect of dicoumarol on AAI-mediated DNA adduct formation, a dose of 20 mg/kg bw of AAI was given by a single *i.p.* injection 2.5 h after the second dose of dicoumarol. Animals were killed 24 h after AAI treatment. Animals in the control groups received the vehicle only. Livers and kidneys were removed after sacrifice, frozen in liquid nitrogen and stored at -80°C until analysis. DNA from livers and kidneys was isolated by extraction with phenol/chloroform (10). Microsomes and cytosols were isolated from the rat tissues by the procedure described previously (18,23). Protein concentration in the microsomal fraction was measured using

bicinchoninic acid protein assay (30) with bovine serum albumin as a standard. Pooled microsomal and cytosolic samples (n = 3 rats/group) were used for the analyses.

DNA adduct analysis by ^{32}P -postlabeling

The nuclease P1 enrichment version of ^{32}P -postlabeling analysis, and thin-layer chromatography (TLC) on polyethylenimine-cellulose (PEI) plates were carried out and DNA adduct levels (RAL, relative adduct labeling) were calculated as described previously (10,31). AA-DNA adducts were identified using reference standards as described (10).

Preparation of antibodies and estimation of CYP1A1, 1A2, and NQO1 protein content in microsomal and cytosolic fractions isolated from rat liver and kidney

The chicken anti-rat CYP1A1, anti-rabbit CYP1A2 and anti-rat NQO1 antibodies were prepared as described previously (32,33). Immunoquantification of microsomal CYP1A1 and 1A2 and cytosolic NQO1 was performed using Western blotting (33). Rat CYP1A1, rabbit CYP1A2 and human NQO1 (Sigma) were used to identify the CYP1A1, 1A2 and NQO1 bands, respectively. The antigen-antibody complex was visualized with an alkaline phosphatase-conjugated rabbit anti-chicken IgG antibody and 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium as dye and bands are expressed as arbitrary units (AU)/mg protein (32,33). Glyceraldehyde phosphate dehydrogenase was used as loading control and detected by its antibody (1:750, Millipore; MA, USA).

NQO1, CYP1A1/2 and 2C6/11 enzyme activity assays

In hepatic and renal cytosols NQO1 activity was measured using menadione (2-methyl-1,4-naphthoquinone) as a substrate; the assay was improved by the addition of cytochrome *c* and NQO1 activity expressed as nmol cytochrome *c* reduced (26,28). Microsomal samples were characterized for specific CYP1A1 and 1A2 activities: ethoxyresorufin *O*-deethylation

(EROD) (CYP1A1/2) and methoxyresorufin *O*-demethylation (MROD) (CYP1A2) (34). CYP1A1 enzyme activity was also monitored by Sudan I hydroxylation to 4'-hydroxy-, 6-hydroxy-, and 4',6-dihydroxy-Sudan I (22). Hepatic microsomal samples were also characterized for specific CYP2C6 and 2C11 activities with their marker substrates determining diclofenac 4'-hydroxylation and testosterone 16 α -hydroxylation, respectively (35,36). In hepatic microsomes POR activity was analyzed using cytochrome *c* as a substrate (24).

Cytosolic and microsomal formation of AAI-DNA adducts

The de-aerated and nitrogen-purged incubation mixtures, in which cytosols were used to activate AAI contained 50 mM Tris-HCl buffer (pH 7.4), 0.2% Tween 20, 1 mM NADPH, 1 mg rat hepatic or renal cytosolic protein, 0.5 mg calf thymus DNA (2 mM dNp) and 0.5 mM AAI in a final volume of 750 μ l. Incubations with cytosols were performed at 37°C for 60 min; AAI-derived DNA adduct formation was found to be linear up to 2 h (18). Control incubations were performed either (i) without cytosol, (ii) without NADPH, (iii) without DNA or (iv) without AAI. After extraction with ethyl acetate DNA was isolated from the residual water phase by the phenol/chloroform extraction method as described (20,23,25).

The de-aerated and nitrogen-purged incubation mixtures, in which microsomes were used to activate AAI contained 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 1 mg of hepatic or renal microsomal protein, 0.5 mg of calf thymus DNA (2 mM dNp) and 0.5 mM AAI in a final volume of 750 μ l. Microsomal incubations were carried out at 37°C for 60 min; AAI-DNA adduct formation was found to be linear up to 2 h in microsomes (23). Control incubations were carried out either (i) without microsomes, (ii) without NADPH, (iii) without DNA or (iv) without AAI. After extraction with ethyl acetate, DNA was isolated from the residual water phase as described above.

Microsomal incubations to study AAI demethylation

Incubation mixtures contained 100 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 1 mg rat hepatic or renal microsomal protein and 10 μ M AAI in a final volume of 250 μ l and were incubated at 37°C for 20 min; AAI *O*-demethylation to AAIA was determined to be linear up to 25 min. Control incubations were carried out either (i) without microsomes, (ii) without NADPH or (iii) without AAI. AAI and its metabolite AAIA were separated by reverse-phase HPLC, identified by mass spectrometry and quantified as described previously (26). Briefly, HPLC was carried out with a Nucleosil 100-5 C₁₈, 25 \times 4.0 mm, 5 mm (Macherey-Nagel) column, using a linear gradient of acetonitrile (20-60% acetonitrile in 55 min) in 100 mM triethylammonium acetate with a flow rate of 0.6 ml/min. A Dionex HPLC pump P580 with UV/VIS UVD 170S/340S spectrophotometer detector set at 254 nm were used. Peaks were integrated with CHROMELEON™ 6.01 integrator. A peak eluting at retention time (r.t.) 22.7 min was identified as AAIA using mass-spectroscopy analysis (26). A typical HPLC chromatogram is shown as a supplementary Figure 1.

Statistical Analyses

For statistical data analysis we used Student's *t*-test. All *P*-values are two-tailed and considered significant at the 0.05 level.

Results

DNA adduct formation in rats treated with dicoumarol and AAI compared to adduct formation in rats treated with AAI alone

AAI-DNA adduct formation was determined by ^{32}P -postlabeling in liver and kidney of rats treated with a single *i.p.* dose of 20 mg/kg bw of AAI. Similarly, rats pretreated orally with total doses of 60 and of 120 mg/kg bw of the NQO1 inhibitor dicoumarol prior to AAI injection were analysed. AAI-DNA adduct patterns in both organs were similar to those found *in vivo* in humans and consisted of two major adducts (spots 1 and 2) and one minor adduct (spot 3) (see Fig. 2, insert) (1,5,10,13). These adducts have been identified to be 7-(deoxyadenosin- N^6 -yl)aristolactam I (dA-AAI; spot 1), 7-(deoxyguanosin- N^2 -yl)aristolactam I (dG-AAI; spot 2) and 7-(deoxyadenosin- N^6 -yl)aristolactam II (dA-AAII; spot 3). No adducts were found in DNA of control rats treated with vehicle only or in those treated with dicoumarol alone (data not shown).

In all rats the levels of AAI-DNA adducts were higher in liver than in kidney, the target organ of AAI genotoxicity (Fig. 2). Interestingly, in contrast to the strong inhibition by dicoumarol observed *in vitro* (18,26), the opposite effect upon AAI-DNA adduct formation was found *in vivo*. In both organs analyzed AAI-DNA adduct levels increased with the dicoumarol dose used in the pretreatment. Compared to adduct levels found in rats treated with AAI alone, DNA binding was 11- and 5.4-fold higher in liver and kidney, respectively, of rats pretreated with a total dose of 120 mg/kg bw of dicoumarol prior to exposure to AAI ($P<0.001$). Therefore, dicoumarol, when administered to rats prior to AAI, seems to induce pathways activating AAI in both organs. Since NQO1 and CYP1A1/2 might determine the AAI-DNA adduct levels their protein levels and enzyme activities were investigated in the two rat organs.

The effect of dicoumarol treatment with or without AAI upon NQO1 and CYP1A1/2 protein levels and their enzymatic activities in rat liver and kidney

Treatment of rats with AAI led to a significant NQO1 protein induction in kidney cytosol (1.4-fold, $p<0.05$), but not in liver. Likewise, a significantly higher NQO1 enzyme activity was found only in kidney of AAI-treated rats (1.5-fold, $p<0.001$). Whereas administration of dicoumarol to rats decreased or did not change NQO1 protein levels in liver, levels increased in a dose-dependent manner in kidney. However, treatment of rats with AAI after pretreatment with dicoumarol resulted in increased NQO1 protein levels in both organs. NQO1 activities in liver cytosols were only significantly higher at the high dicoumarol dose in combination with AAI, whereas they were significantly higher in kidney cytosols from rats treated with AAI, dicoumarol alone or dicoumarol with AAI (Fig. 3).

The effect of exposure to AAI and dicoumarol on the protein levels of microsomal CYP1A1/2 in rats was also examined as both CYP enzymes play a dual role in AAI metabolism. In control rats higher protein levels of CYP1A1 were found in hepatic than in renal microsomes by Western blotting (Fig. 4A,B). These levels corresponded with the CYP1A enzyme marker activities; EROD for CYP1A1/2 (Fig. 5 C,D) and Sudan I oxidation for CYP1A1 (Fig. 5 G,H). As shown in Figures 4 and 5 dicoumarol treatment alone, and particularly in combination with AAI, resulted in lower hepatic CYP1A1 protein levels but this was not reflected in decreased enzyme activity (i.e. EROD and Sudan I oxidation). In contrast, statistically significant ~3-fold higher CYP1A1 protein levels were found in kidneys of rats treated with dicoumarol relative to control rats. This was paralleled by Sudan I oxidation and this difference was even more pronounced when measuring EROD activities (Figs. 4 and 5). AAI also induced renal CYP1A1 protein levels and activities which was further enhanced in combination with the lower dicoumarol dose, but did not reach the levels induced by dicoumarol alone (Figs. 4 and 5).

CYP1A2 was found to be expressed only in rat liver, and not in kidney (Fig. 4D) confirming earlier studies showing that CYP1A2 is an almost exclusively hepatic enzyme (37). In concordance, MROD activity, a marker reaction of CYP1A2, was found in liver, but was negligible in kidney (Fig. 5E,F). AAI and dicoumarol treatment alone as well as dicoumarol pretreatment prior to AAI administration resulted in elevated CYP1A2 protein levels and MROD activities in the liver (Figs. 4C and 5E), except in the rats treated with 120 mg/kg bw of dicoumarol prior to AAI administration; CYP1A2 protein level remained while MROD activity increased 2-fold. This suggests that the weak CYP1A2 signals detected by Western blotting probably do not truly reflect protein levels and determination of enzyme activity provides a more accurate assessment of enzyme induction.

The activity of POR was increased in rat kidney by AAI or dicoumarol treatment alone as well as in combined administration, but remained essentially unchanged in liver (Fig. 6). POR not only acts as an electron donor in catalytic functions of CYPs (38,39), but is also able to activate AAI to some extent (23).

Cytosolic versus microsomal activation of AAI

In further experiments AAI-DNA adduct formation catalyzed by cytosols isolated from liver and kidney of rats from all treatment groups was investigated *ex vivo*. Cytosols were incubated with AAI, DNA and the cofactor of NQO1, NADPH, and analyzed for DNA adduct formation by ³²P-postlabeling. AAI was activated by the cytosols from both organs as evidenced by AAI-DNA adduct formation (Fig. 7 A, B) and the DNA adduct pattern was the same as those found *in vivo* (see Fig. 2, insert). No adducts were observed in control incubations carried out in parallel (data not shown). In kidney cytosols from all modes of treatment AAI-DNA adduct levels increased up to 3.3-fold relative to cytosols isolated from untreated animals (controls). Renal cytosols isolated from AAI-treated rats led to 2.5-fold higher adduct levels than cytosol from control animals ($p < 0.001$) (Fig. 7B) and corresponded

to higher NQO1 protein levels in these cytosolic samples (compare Figs. 3 and 7). Despite a lack of significant NQO1 induction at either protein or activity levels by AAI, DNA adduct levels catalyzed by rat hepatic cytosols were 1.7-fold higher than in controls (Fig 3). These results indicate that other cytosolic nitroreductases may contribute to the increased AAI-DNA adduct formation observed in rat liver cytosol. In this context it is noteworthy that previous studies have shown that xanthine oxidase is capable of activating AAI (18,19).

Consistent with the lower NQO1 protein and activity levels elicited by dicoumarol in liver (see Fig. 3A and C), AAI-DNA adduct formation was lower in hepatic cytosol isolated from rats treated with 60 mg/kg bw of dicoumarol. However, when rats were treated with dicoumarol prior to AAI administration, AAI-DNA adduct levels increased up to 2.6-fold relative to hepatic cytosolic incubations from control rats (Fig. 7A). In kidney cytosols from all modes of treatment AAI-DNA adduct formation was up to 3.3-fold higher relative to controls.

Because microsomal CYP1A1/2 are also able to activate AAI by nitroreduction to species forming DNA adducts, hepatic and renal microsomes of control and treated rats were analyzed for their capacity to form AAI-DNA adducts in *ex vivo* incubations. AAI was reductively activated by hepatic and renal microsomes from all rats (Fig. 7C,D). The DNA adduct pattern generated was the same as that found *in vivo* (see Fig. 2, insert). No adducts were observed in control incubations carried out in parallel (data not shown). The only significant increase in AAI-DNA adduct levels catalyzed by hepatic microsomes was seen in the two groups exposed to 60 mg/kg bw dicoumarol (Fig. 7C). AAI-DNA adduct formation was up to 2.7-fold higher in kidney microsomes from all treatment groups relative to controls; except by those isolated from rats pretreated with 120 mg/kg bw dicoumarol prior to AAI administration (Fig. 7D). The reason for such a finding remains to be explained.

The effect of treatment of rats with AAI and dicoumarol as well as pretreatment with dicoumarol prior to AAI administration on oxidation of AAI to AAIA by rat hepatic and renal microsomes

Since microsomal CYP1A1/2 also detoxify AAI to its *O*-demethylated metabolite AAIA (23-25,40), AAIA formation by hepatic and renal microsomes was investigated *ex vivo*. Under the conditions used, liver and kidney microsomes oxidized AAI to AAIA (Fig. 5A,B). A dose-dependent decrease in AAI oxidation to AAIA in hepatic and renal microsomes (up to ~40% relative to controls) was caused by treatment of rats with dicoumarol or pretreatment of rats with dicoumarol prior to AAI administration. However, this decrease did not correspond to CYP1A enzyme activities shown in Figures 5C and 5D. In order to explain this discrepancy, we further investigated the effect of dicoumarol on the oxidation of AAI to AAIA catalyzed by rat hepatic microsomes. We found that AAIA formation was strongly inhibited by dicoumarol (with an IC₅₀ value of 2 µM), but CYP1A enzyme activity as measured by EROD, MROD and Sudan I oxidation did not change (data not shown). Collectively these results suggest that not only CYP1A1/2, but also other CYPs might contribute to the oxidation of AAI to AAIA in rat hepatic microsomes and that these enzymes are modified by dicoumarol. We hypothesized that CYPs of the 2C subfamily might be candidates as they can also oxidize AAI to AAIA to some extent (26) and because CYP2C enzymes are highly expressed in the livers of male rats accounting for approximately 55% of the rat liver CYP complement (41). Among them CYP2C11 and 2C6 contribute about 50% and 20% to the total hepatic CYP2C content in rats, respectively (42,43), and both CYP2C isoenzymes have previously been shown to be able to *O*-demethylate AAI to AAIA (26). To test this hypothesis the effect of dicoumarol treatment *in vivo* upon CYP2C6 and 2C11 activities in hepatic microsomes was analyzed (Fig. 8); diclofenac 4'-hydroxylation and testosterone 16 α -hydroxylation were used as marker activities for CYP2C6 and 2C11, respectively (35,36). As shown in Figure 8 exposure of rats to dicoumarol, either with or without AAI, decreased testosterone 16 α -hydroxylation

activities up to 60% relative to control; diclofenac 4'-hydroxylation was reduced up to 20%. Therefore, the lower CYP2C enzyme activities could explain the lower oxidation rates of AAI to AAIIa in these microsomes and indicate that CYP2C11 and 2C6 can contribute to AAIIa formation in rat liver.

Discussion

Previously we have demonstrated that NQO1 expressed in human, rat or mouse liver and kidney as well as purified human and rat NQO1 is the predominant enzyme responsible for the genotoxicity of AAI *in vitro* (18-21). In addition, other studies suggested that NQO1 might also contribute to AAI-DNA adduct formation *in vivo* (19-21,24,26,28). In the present study we utilized dicoumarol, an inhibitor of NQO1, in a Wistar rat animal model and showed that the contribution of NQO1 to AAI activation is complex when comparing *in vitro* and *in vivo* findings. Bioavailability of AAI to different organs, species differences, length of exposure to dicoumarol and AAI, and the applied doses seem to be crucial parameters in the effect of this NQO1 inhibitor upon AAI genotoxicity.

Dicoumarol had previously been administered to male C57BL/6 mice to investigate the participation of NQO1 in AAI metabolism and AAI-induced nephrotoxicity (22). The authors found that NQO1 indeed plays a crucial role in (renal) AAI nitro-reduction *in vivo*. They showed that NQO1 activity was inhibited in a concentration-dependent manner in mice treated with increasing doses of dicoumarol. A single *i.p.* injection of AAI 2.5 h after dicoumarol pretreatment resulted in decreased levels of its reductive metabolite aristolactam I in kidney. As a consequence levels of AAI, and particularly AAIA, in serum and kidney were higher in dicoumarol-pretreated mice. Furthermore, pretreatment with this NQO1 inhibitor decreased AAI-induced nephrotoxicity and increased the survival rate of these mice (22). Collectively, these results suggested that dicoumarol acts as an inhibitor of AAI reduction in this mouse model.

The aim of the present study was to evaluate the formation of AAI-DNA adducts in rats exposed to AAI in the presence of two concentrations of dicoumarol. We administered the higher doses of dicoumarol and AAI and the same dosing schedule as Chen *et al.* (22) that had been used for mice, and AAI-DNA adduct formation was monitored as the biological end-point of AAI genotoxicity. The resultant adduct levels obtained *in vivo* and *ex vivo*

incubations were used as a surrogate measure of the activity of enzymes involved in AAI bioactivation. Previous studies in rats have shown that dicoumarol is still present up to 10 h after oral administration and concentrations probably increasing in blood due to the slow uptake from the intestine (44). In addition dicoumarol has a long elimination half-life ranging from 5-25 h in rats (45). Here we found that AAI-DNA adduct levels in kidney, and particularly in liver, were higher in rats treated with dicoumarol prior to AAI administration (Figs. 2 and 9). This finding is unexpected since NQO1, the target of dicoumarol inhibition, is the primary AAI activating enzyme in rats, mice and humans.

Hepatic cytosols from rats treated with dicoumarol showed the same NQO1 protein levels as those from untreated rats, whereas the enzyme activities were decreased ~2-fold. This may be due to dicoumarol still binding to NQO1 as dicoumarol has strong protein binding properties. It is expected that due to its long elimination half-life from blood residual dicoumarol may remain in tissues, particularly in liver as its main site of metabolism (45). The decreased NQO1 enzyme activity resulted in lower DNA adduct formation in *ex vivo* incubation with AAI using hepatic cytosols. The situation in the kidney was more complex. Because the effect of residual dicoumarol upon enzyme activity *ex vivo* was not observed, overall dicoumarol concentrations might be lower in the kidney compared to liver (45). In kidney dicoumarol induced NQO1 protein levels and also activity. Furthermore, in liver and kidney NQO1 protein levels and activity was induced by treating rats with dicoumarol prior to AAI administration and this corresponded to higher levels of AAI-DNA adducts formed *in vivo* and in *ex vivo* incubations of AAI with cytosols of these organs.

Higher adduct levels found in liver and kidney of rats pretreated with dicoumarol prior to AAI administration might be, beside increased levels and activities of NQO1, also the result of higher protein levels and activities of CYP1A1 in kidney and CYP1A2 in liver. Namely, the two CYP1A enzymes can reductively activate AAI to DNA binding (24-26). However, an increase in the levels of AAI-DNA adducts formed in *ex vivo* incubations of

AAI with hepatic and renal microsomes from rats treated with dicoumarol prior to AAI was lower than that generated in *ex vivo* incubations of AAI with cytosols from these rats (see Fig. 7). The reductive activation of AAI by these two CYP1A enzymes might therefore be less important for an increase in AAI-DNA adduct levels in rat liver and kidney *in vivo* than induction of NQO1. Furthermore, oxidation of AAI by both CYPs was found to lead to AAIA formation (Fig. 1) (24-26). In the present study the detoxication metabolite AAIA generated in *ex vivo* incubations was lower in microsomes isolated from either kidney or liver from rats treated with dicoumarol relative to controls. Here again as in the case of NQO1 we expect dicoumarol to still be present in hepatic microsomes (45). The low IC₅₀ value of 2 μ M dicoumarol determined for the inhibition of AAIA formation in hepatic microsomes shows that only small amounts of dicoumarol need to be bound to affect AAI demethylation. This is in strong contrast to the observation of increased AAIA serum levels in dicoumarol-pretreated mice administered AAI (22). This would point to another enzyme system or another organ demethylating AAI *in vivo*, at least in mice. Since dicoumarol inhibition of AAIA formation in hepatic microsomes did not correlate with CYP1A1/2 activity, other CYPs were considered. We examined the specific activities of two members of the rat CYP2C subfamily also known to *O*-demethylate AAI to AAIA (26). Indeed both CYP2C11 and 2C6 enzyme activities were inhibited by dicoumarol; the same effect was also seen in combination with AAI treatment. Dicoumarol itself is mainly metabolized in the liver and earlier work showed that human CYP2C is responsible (46), although it is noteworthy that this finding has not been further investigated. The authors used antibodies generated against rat CYP2C7 and 2C11 and found that formation of 7-hydroxy-dicoumarol in human hepatic microsomes was inhibited by 50-60% (46). Collectively, these data suggest a competitive inhibition of CYP2C activity by residual dicoumarol in our *ex vivo* system. In addition, an inhibition of *O*-demethylation of AAI to AAIA was even higher when rats were treated with dicoumarol and AAI (see Fig. 5A).

All these findings indicate that higher adduct levels found in the liver of rats pretreated with dicoumarol prior to AAI administration might be, beside induction of NQO1, also caused by decreased AAI detoxication to AAIa due to CYP2C inhibition by dicoumarol and AAI. Higher levels of AAI are available for its reductive activation to form AAI-DNA adducts (Fig. 9). In kidney induction of NQO1 with dicoumarol and AAI seems to be the predominant process responsible for higher levels of AAI-DNA adducts in this organ (Fig. 9).

The inductive effect of AAI on NQO1 found in this work confirmed previous studies, where NQO1 protein levels and its enzyme activity were induced by AAI in kidney of mice (26,28,29). Thus, NQO1 might also be induced in the kidneys of patients suffering from AAN and BEN, and this feature can contribute to an elevated risk for cancer. However, in this study we found for the first time that dicoumarol also induces the NQO1 protein and its enzyme activity in the AAI target organ kidney. Because dicoumarol is used in medicine as an anticoagulant drug that functions as a vitamin K antagonist (similar to warfarin for which it was the inspiration) [reviewed in (47,48)], such an induction might increase AAI-DNA adduct formation leading to a higher risk of AAI-mediated development of urothelial cancer in humans exposed to AA.

Even though we demonstrate in the present study that both AAI and dicoumarol increased NQO1 protein levels and activity, the mechanism(s) are not yet clear. NQO1 induction has been widely investigated in a variety of studies [reviewed in (49-53)]. Protein levels of NQO1 are induced by several chemicals, often by pathways generating reactive oxygen species (ROS). *NQO1* gene expression is primarily regulated by the KEAP1/NRF (NF-E2-related factor 2) pathway, which controls redox homeostasis and facilitates the adaptation of most cells to oxidative stress (50-53.). Because ROS have been found in some human cells treated with AAI (54,55), ROS formation caused by AAI might contribute to NQO1 induction.

NQO1 is known to be implicated in vitamin K metabolism; it is identical to the so-called dicoumarol-inhibited vitamin K reductase (53,56). Because vitamin K is redox-cycled during its metabolism by other enzymes, ROS might be generated in the one-electron redox reactions in this metabolism (53,57). Since NQO1 competes with enzymes that redox cycle vitamin K and catalyzes two-electron reduction of vitamin K, less semiquinone and ROS are formed. If dicoumarol inhibits NQO1 activity, a significant increase in ROS might occur (53,56) also resulting in enhanced *NQO1* expression. Because our data in rats and the results of the mouse study (22) do not unequivocally answer the question how important NQO1 is for AAI activation *in vivo*, utilization of *NQO1*-knockout animal models (58) may bring a definite proof. The elucidation of the contribution of NQO1 to AAI bioactivation resulting in its genotoxicity and nephrotoxicity is of great importance. The activity of NQO1 in humans may differ significantly among individuals, because, beside its inducibility, the gene is polymorphic. Therefore, the impact of *NQO1* genotype on AAI-induced nephropathy and urothelial cancer in humans remains still to be clarified. We propose that analyses of the expression levels and activities of enzymes metabolizing AAI in AAN and BEN patients may clarify to which extent they contribute to the development of AA-induced nephropathies and cancer.

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Legends to Figures

Figure 1. Pathways of biotransformation and DNA adduct formation of AAI. dA-AAI, 7-(deoxyadenosin- N^6 -yl)aristolactam I; dG-AAI, 7-(deoxyguanosin- N^2 -yl)aristolactam I; NR, nitro-reduction; Uridine-diphosphate- β -D-glucuronosyltransferase; SULT, sulfotransferase.

Figure 2. Quantitative TLC 32 P-postlabeling analysis of AAI-DNA adduct levels in organs of rats treated *i.p.* with a single dose of 20 mg/kg bw AAI with or without the pretreatment with dicoumarol (total doses of 60 [DC60] or 120 mg [DC120] dicoumarol/kg bw) (see Materials and Methods for details). Numbers above columns ("F") indicate fold changes in DNA adduct levels in AAI-treated animals pretreated with dicoumarol compared to animals treated with AAI alone. Values are given as the means \pm SD ($n = 3$); each DNA sample was determined by two postlabeled analyses. RAL, relative adduct labelling. Comparison was performed by *t*-test analysis; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, different from animals treated with AAI alone. Insert: autoradiographic profile of AA-DNA adducts in rat liver using the nuclease P1 enrichment version of the assay. The adduct profile shown is representative of those obtained in other organs investigated. The origin, in the bottom left-hand corner, was cut off before exposure. Spot 1, dA-AAI; spot 2, dG-AAI; and spot 3, dA-AAII.

Figure 3. NQO1 enzyme activity (A, B) and NQO1 protein levels (C, D) in rat cytosols isolated from untreated (control) animals and animals pretreated orally with dicoumarol (total doses of 60 [DC60] or 120 mg [DC120] dicoumarol/kg bw) alone or in combination with AAI (single *i.p.* dose of 20 mg/kg bw). NQO1 activity in hepatic (A) and renal cytosols (B) was determined using menadione and cytochrome *c* as substrate, (expressed as nanomoles cytochrome *c* per minute per milligram protein). Cytosol isolated from liver (C), or kidney (D) was analyzed by Western blotting in the same blot (insert) and,

therefore, can be compared directly. Human recombinant NQO1 (Sigma) was used to identify the rat NQO1 band in rat cytosol (data not shown). Numbers above columns (“F”) indicate fold changes in protein level or enzyme activity compared to control. Values are given as the means \pm SD ($n = 3$). Comparison was performed by *t*-test analysis; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, different from control.

Figure 4. CYP1A1 (A, B) and CYP1A2 (C, D) protein levels in rat microsomes isolated from untreated (control) animals and animals pretreated orally with dicoumarol (total doses of 60 [DC60] or 120 mg [DC120] dicoumarol/kg bw) alone or in combination with AAI (single *i.p.* dose of 20 mg/kg bw). Microsomes isolated from liver (A, C) and kidney (B, D) were analyzed by Western blotting in the same blot (insert) and therefore can be directly compared. Rat recombinant CYP1A2 was used to identify the rat CYP1A2 band (see panel D). Values are given as the means of arbitrary units (AU) \pm SD ($n = 3$). Numbers above columns (“F”) indicate fold changes in protein level or enzyme activity compared to control. ND, not detected. Comparison was performed by *t*-test analysis; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, different from control.

Figure 5. AAI oxidation to AAIa and CYP1A enzyme activities in rat microsomes isolated from untreated (control) animals and animals pretreated orally with dicoumarol (total doses of 60 [DC60] or 120 mg [DC120] dicoumarol/kg bw) alone or in combination with AAI (single *i.p.* dose of 20 mg/kg bw). Formation of AAIa (peak area per minute per milligram protein) from AAI in hepatic (A) and renal (B) microsomes. CYP1A enzyme activity as measured by EROD activity (picomoles resorufin per minute per milligram protein) (C, D), MROD activity (picomoles resorufin per minute per milligram protein) (E, F) or Sudan I oxidation (nanomoles total C-hydroxylated metabolites per minute per milligram protein) (G, H). All values are given as the means \pm SD ($n = 3$). Numbers above columns (“F”) indicate fold changes in AAIa levels or enzyme activities compared

to control. ND, not detected. Comparison was performed by *t*-test analysis; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, different from control.

Figure 6. POR enzymatic activity in rat microsomes isolated from untreated (control) animals and animals pretreated orally with dicoumarol (total doses of 60 [DC60] or 120 mg [DC120] dicoumarol/kg bw) alone or in combination with AAI (single *i.p.* dose of 20 mg/kg bw). POR was measured using cytochrome *c* as substrate (nanomoles cytochrome *c* per minute per milligram protein). All values are given as the means \pm SD (*n* = 3). Numbers above columns ("F") indicate changes in enzyme activity compared to control. Comparison was performed by *t*-test analysis; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, different from control.

Figure 7. DNA adduct formation *ex vivo* by AAI in rat cytosols (A, B) and microsomes (C, D) isolated from untreated (control) animals and animals pretreated orally with dicoumarol (total doses of 60 [DC60] or 120 mg [DC120] dicoumarol/kg bw) alone or in combination with AAI (single *i.p.* dose of 20 mg/kg bw). AAI-DNA adduct formation was determined by ³²P-postlabeling in hepatic (A, C) and renal (B, D) fractions. Values are given as the means \pm SD (*n* = 3); each DNA sample was determined by two postlabeling analyses. RAL, relative adduct labeling. Numbers above columns ("F") indicate fold changes in DNA adduct levels compared to control. Comparison was performed by *t*-test analysis; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, different from control.

Figure 8. CYP2C11 (A) and 2C6 enzyme activities (B) in rat hepatic microsomes isolated from untreated (control) animals and animals pretreated orally with dicoumarol (total doses of 60 [DC60] or 120 mg [DC120] dicoumarol/kg bw) alone or in combination with AAI (single *i.p.* dose of 20 mg/kg bw). CYP2C11 was measured as testosterone 16 α -hydroxylation (nanomoles 16 α -hydroxytestosterone per minute per milligram protein) and CYP2C6 as diclofenac 4'-hydroxylation (nanomoles 4'-hydroxydiclofenac per minute per milligram protein). All values are given as the means \pm SD (*n* = 3). ##. Sample

lost. Numbers above columns (“F”) indicate fold changes in enzyme activities compared to control. Comparison was performed by *t*-test analysis; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, different from control.

Figure 9. Schematic summary showing the effects of dicoumarol treatment in rats on AAI metabolism. NQO1 induction with dicoumarol prior to AAI administration led to increased AAI-DNA adduct formation catalyzed by rat hepatic and renal cytosols (see panel showing AAI-DNA adduct levels *in vitro*). Inhibition of CYP2C by dicoumarol treatment led to a decrease of AAIa formation in hepatic microsomes (see panel showing AAI oxidation *in vitro*). Both NQO1 induction and CYP2C inhibition increase AAI-DNA adduct formation in liver, while only NQO1 induction impacts on AAI-DNA binding in kidney (see panels showing AAI-DNA adduct levels *in vivo*). “F” indicates fold increase in rats pretreated with dicoumarol compared to animals treated with AAI alone. Comparison was performed by *t*-test analysis; ***P* < 0.01, ****P* < 0.001. AAI - aristolochic acid I; DC – dicoumarol; CYP – cytochrome P450; NQO1 – NAD(P)H:quinone oxidoreductase 1.

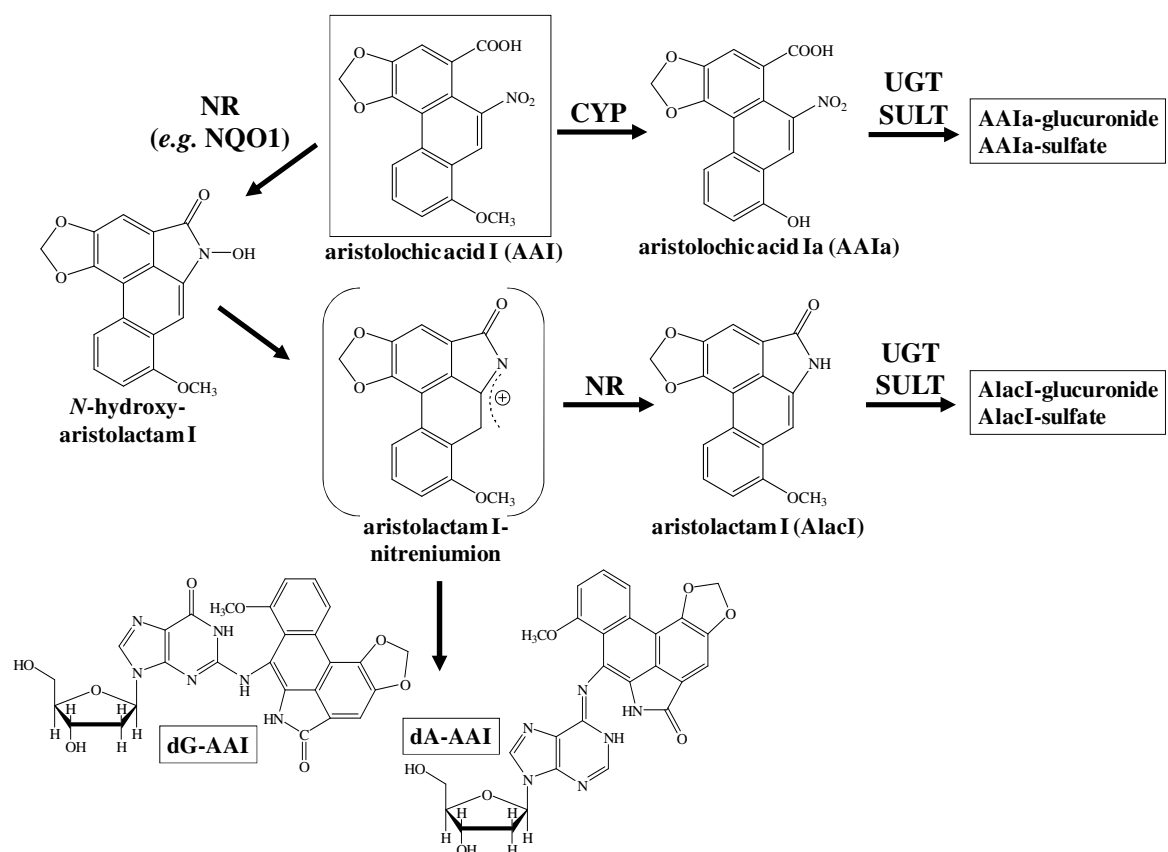


Figure 1

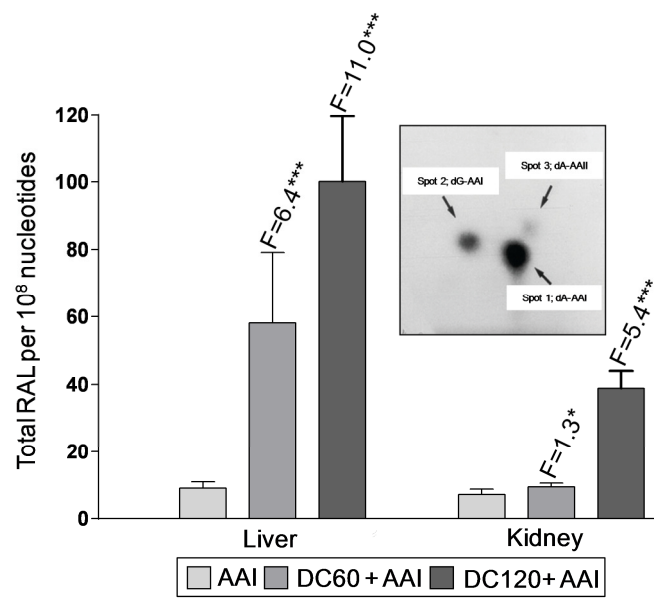


Figure 2

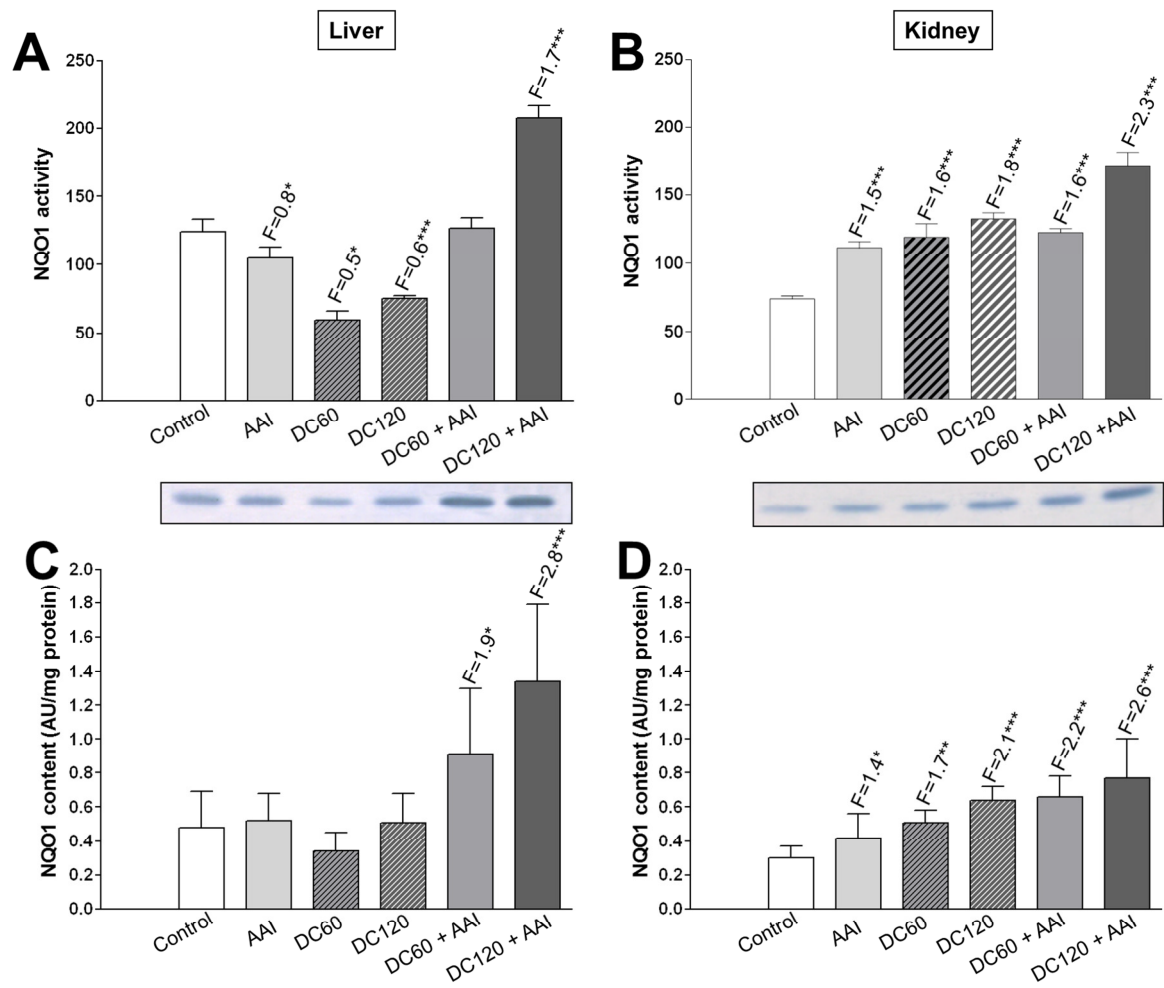


Figure 3

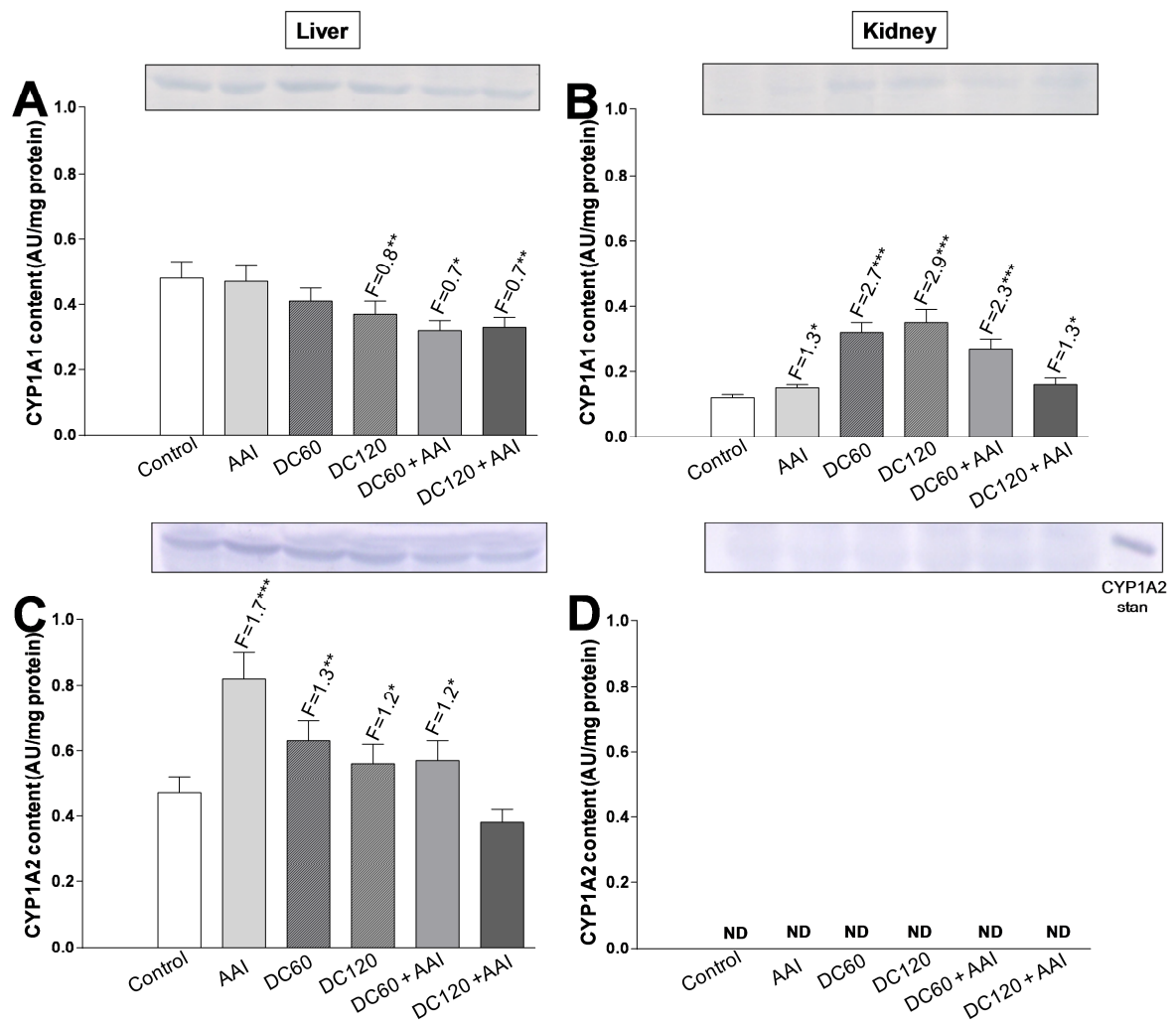


Figure 4

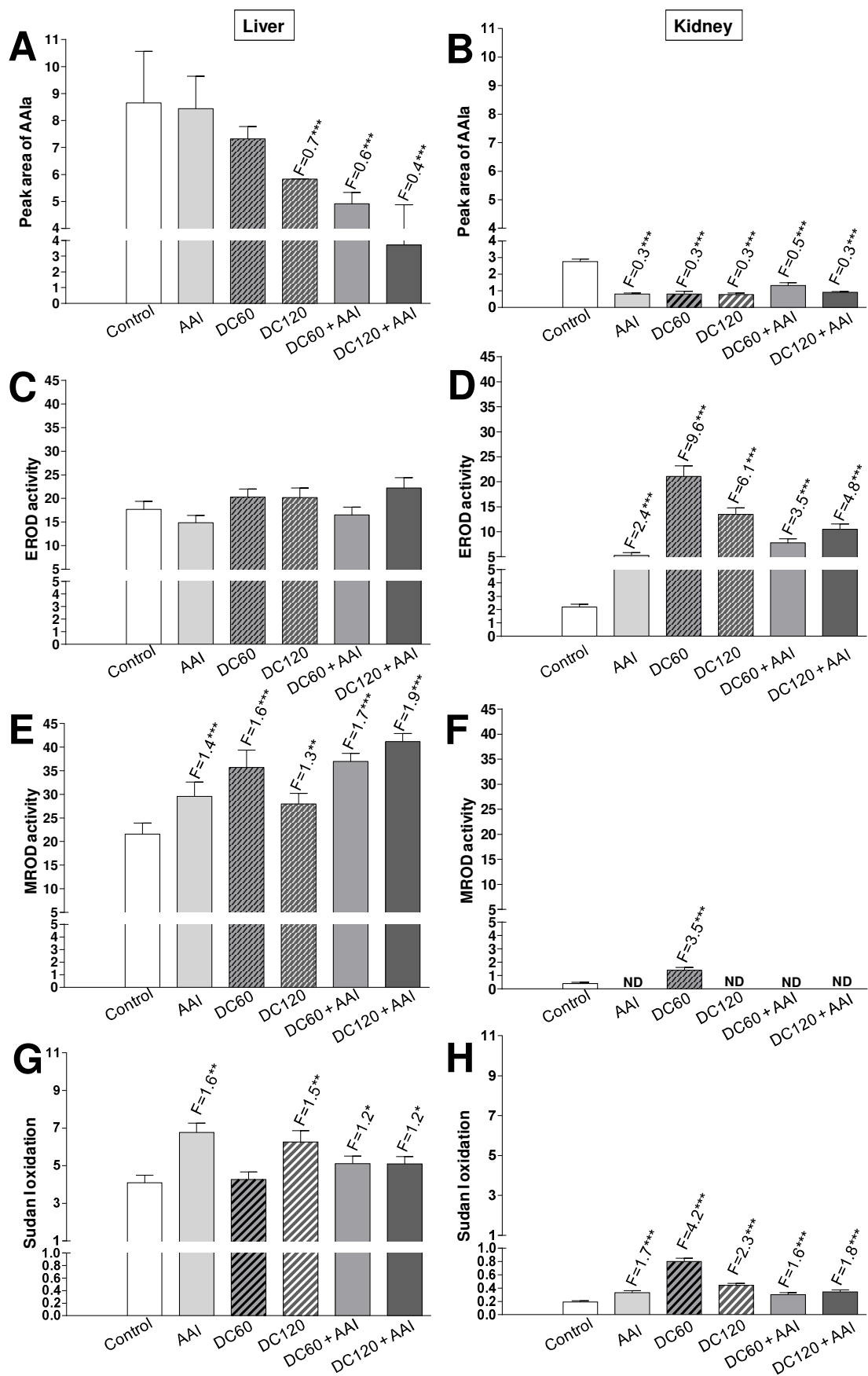


Figure 5

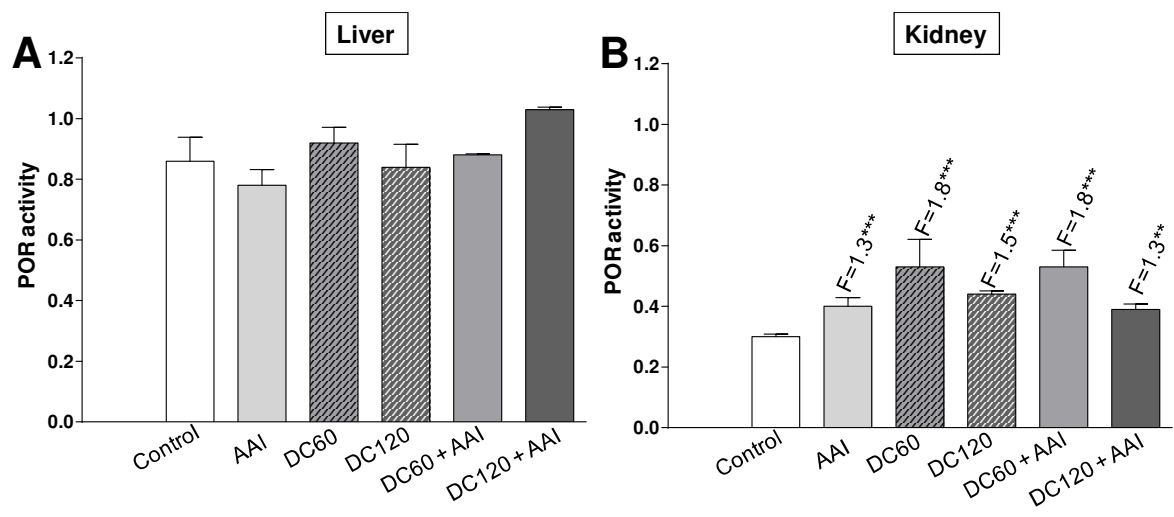


Figure 6

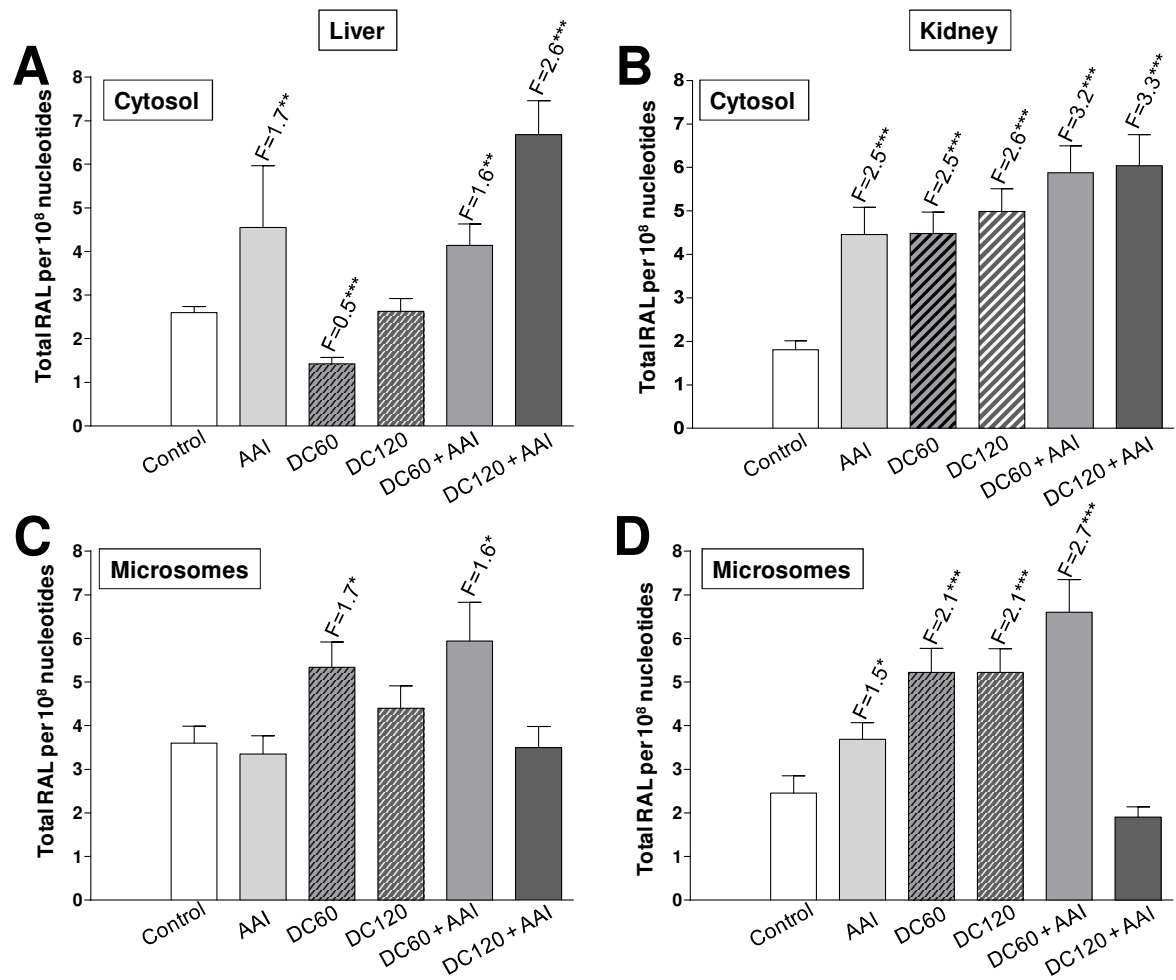


Figure 7

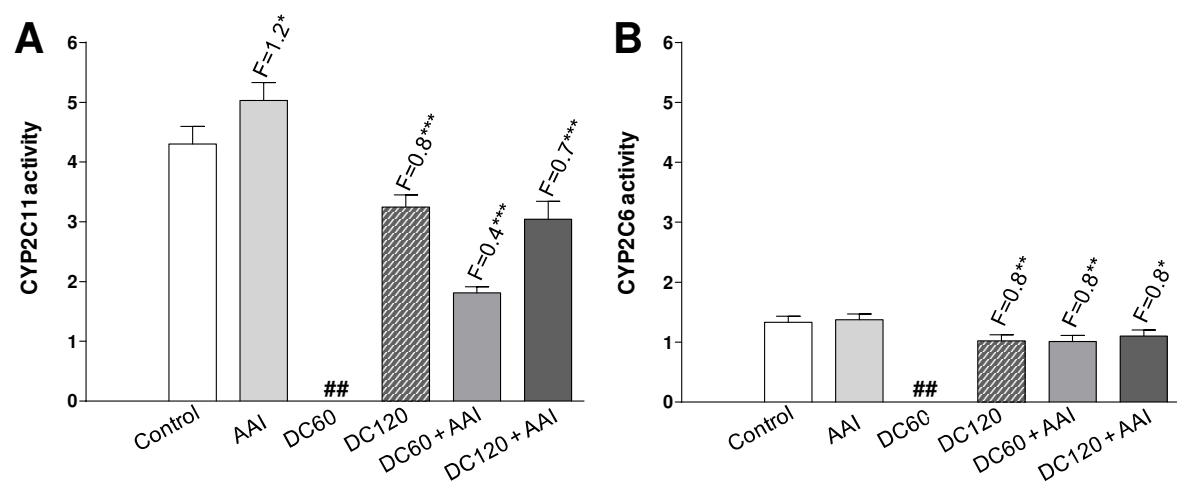


Figure 8

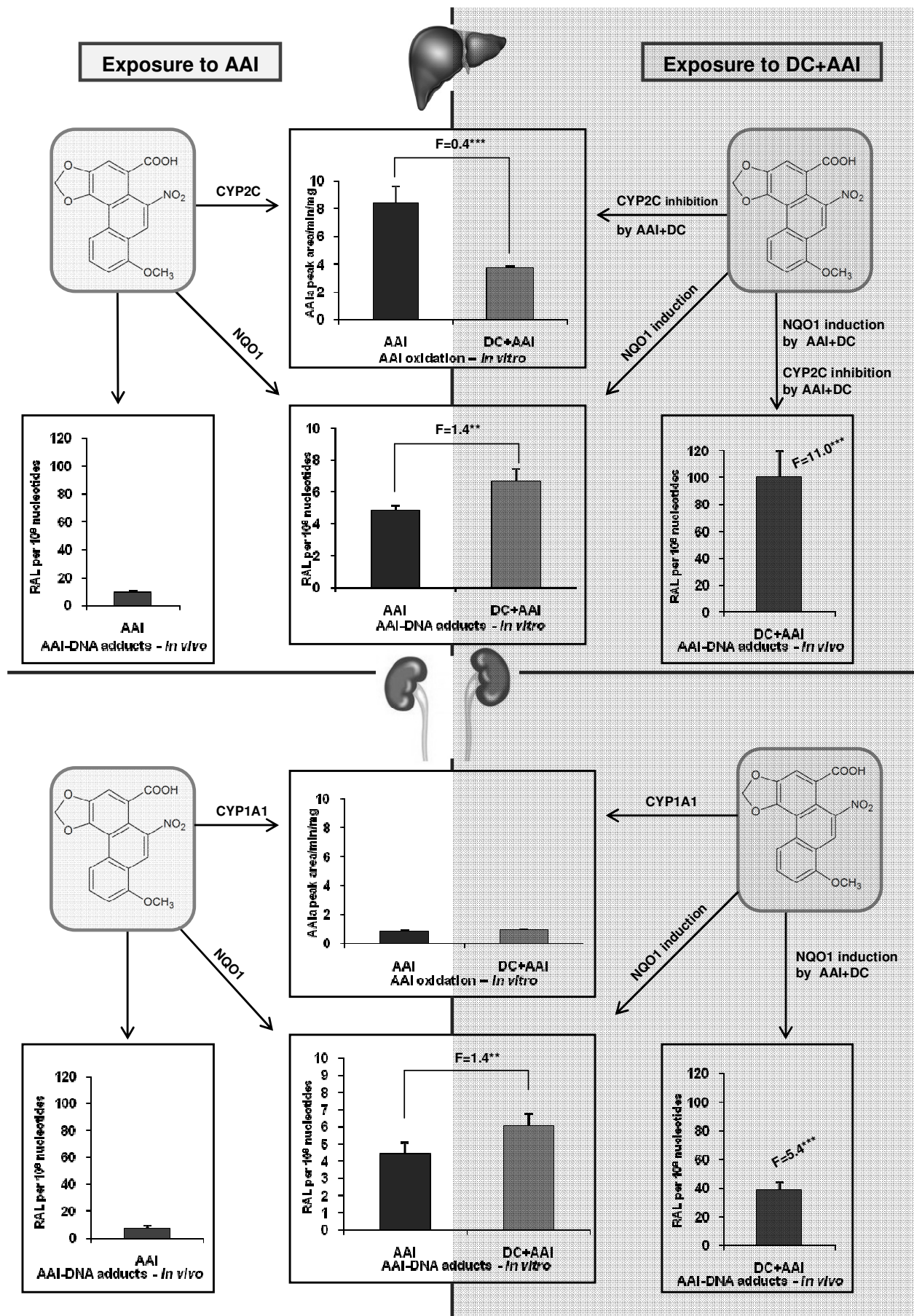


Figure 9